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Chen, Q., Soundara Pandi, S. P., Kerrigan, L., McElvaney, N. G., Greene, C. M., Elborn, J. S., Taggart, C. C., & Weldon, S. (2018). Cystic fibrosis epithelial cells are primed for apoptosis as a result of increased Fas (CD95). *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, 17(5), 616-623.
<https://doi.org/10.1016/j.jcf.2018.01.010>

Published in:

Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society

Document Version:

Peer reviewed version

Queen's University Belfast - Research Portal:

[Link to publication record in Queen's University Belfast Research Portal](#)

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Cystic Fibrosis Epithelial Cells are Primed for Apoptosis as a Result of Increased Fas (CD95)

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Abstract

Background: Previous work suggests that apoptosis is dysfunctional in cystic fibrosis (CF) airways with conflicting results. We evaluated the relationship between dysfunctional cystic fibrosis transmembrane conductance regulator (CFTR) and apoptosis in CF airway epithelial cells.

Methods: Apoptosis and associated caspase activity were analysed in non-CF and CF tracheal and bronchial epithelial cell lines.

Results: Basal levels of apoptosis and activity of caspase-3 and caspase-8 were significantly increased in CF epithelial cells compared to controls, suggesting involvement of extrinsic apoptosis signalling, which is mediated by the activation of death receptors, such as Fas (CD95). Increased levels of Fas were observed in CF epithelial cells and bronchial brushings from CF patients compared to non-CF controls. Neutralisation of Fas significantly inhibited caspase-3 activity in CF epithelial cells compared to untreated cells. In addition, activation of Fas significantly increased caspase-3 activity and apoptosis in CF epithelial cells compared to control cells.

Conclusions: Overall, these results suggest that CF airway epithelial cells are more sensitive to apoptosis via increased levels of Fas and subsequent activation of the Fas death receptor pathway, which may be associated with dysfunctional CFTR.

Keywords: Epithelial cells, Apoptosis, Fas

Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. It affects all exocrine organs including the airways, pancreas, intestine and sweat glands, however, pulmonary disease causes most of the morbidity and mortality associated with CF [1]. CFTR – a 1480 amino acid protein that functions as a chloride channel – is highly expressed in the airway epithelium where it regulates chloride ion movement [2]. A single deletion of a phenylalanine residue at position 508 in CFTR ($\Delta F508$ -CFTR) results in a misfolded protein that is associated with approximately 90% of CF cases [3]. CF lung disease is associated with the failure of pulmonary host defence leading to a vicious cycle of continual infection, inflammation and remodelling of lung tissue. The airway epithelium plays a key role in pulmonary host defence, and apoptosis is a physiological process essential for homeostasis of epithelial function.

Apoptosis, or programmed cell death, is a form of regulated cell death in which activation of specific proteases called caspases leads to DNA cleavage and cell death. Previous work suggests that apoptosis is dysfunctional in the CF airways with conflicting results. While some reports describe defective apoptosis of epithelial cells expressing mutant forms of CFTR [4–6], a number of others report excessive apoptosis in CF cells [7–13]. In addition, it is unclear how CFTR misfolding and dysfunction contributes to apoptosis or the susceptibility of cells to pro-apoptotic stimuli. Nonetheless, accumulation of apoptotic cells as evident in the CF lung may precipitate chronic inflammation and progressive airway damage [14,15].

Previous studies have focused on the effect of external stimuli on apoptosis in CF epithelium [8,16]. However, we now show that in basal (unstimulated) CF epithelial cells, indices of apoptosis are increased (caspase-3, caspase-8). Further evaluation of upstream apoptotic factors demonstrated increased expression of Fas which contributed to the increased activation of caspase-3 and -8 demonstrated in these cells. Treatment of CF cells with Fas activating antibody, CH-11, resulted in a significant increase in caspase-3 and -8 activation as well as significant increase in apoptosis in these cells compared to non-CF cells. Increased Fas expression was related to CFTR function as treatment of primary non-CF bronchial epithelial cells with a CFTR inhibitor resulted in increased Fas expression in these cells. These findings indicate that CF epithelium is in a primed condition for apoptosis which results in significant programmed cell death upon activation. These finding may impact on effective innate host defence function offered by the epithelial barrier in the CF lung.

Methods and materials

Cell culture

The human bronchial epithelial cell line (16HBE14o-, HBE), CF bronchial epithelial cell line (CFBE41o-, CFBE) homozygous for the $\Delta F508$ mutation, the human tracheal epithelial cell line (9HTEo-, HTE) and the CF tracheal epithelial cell line (CFTE29o-, CFTE) homozygous for the $\Delta F508$ mutation were obtained as a gift from Prof Dieter Gruenert (California Pacific Medical Center Research Institute, San Francisco, CA, USA). Cell lines were maintained in Minimum Essential Medium supplemented with 10% heat-inactivated foetal calf serum, 2mM L-glutamine and 1% penicillin/streptomycin (PAA Laboratories, Somerset, UK) at 37°C under 5% CO₂. Normal human bronchial/tracheal epithelial cells (NHBE) were obtained from Lonza (Basel, Switzerland) and were cultured according to provided guidelines.

For experiments, non-CF (HBE, HTE) and CF epithelial cell lines (CFBE, CFTE) were adhered overnight. Cell-free supernatants were collected following a further 24 hr in fresh media. Whole cell lysates for protease activity assays and Western blotting were prepared as described below. Total RNA was isolated from cells using TRI Reagent® (Sigma-Aldrich) as per manufacturer's instructions.

Cell treatments

For caspase-8 inhibitor experiments, CF epithelial cells (CFTE, CFBE) were left untreated or treated with Ac-IETD-CHO (10 μ M, Enzo Life Sciences, Inc., Exeter, UK) for 24 hr. For CFTR inhibitor experiments, non-CF epithelial cells (HTE, HBE, NHBE) were treated \pm CFTR inhibitor (CFTR_{inh-172}, 10 - 20 μ M) for 24 hr. For Fas activation experiments, non-CF and CF epithelial cells were treated \pm a human Fas activating antibody (clone CH-11, 0.5 μ g/ml,

Millipore, Darmstadt, Germany) for 24 hr. For Fas neutralisation experiments, cells were treated with 100 ng/ml anti-Fas neutralising antibody (clone ZB4, Millipore) or corresponding isotype control mouse (IgG₁, R&D Systems Europe Ltd., Abingdon, UK) for 6 hr.

Bronchial brushing analysis and study approval

Following informed consent under a protocol approved by Beaumont Hospital Institutional Review Board, bronchial brushings were sampled from individuals with CF (n = 3) and non-CF controls (n=3). Total RNA was isolated and reverse transcribed as previously described [17].

Apoptosis detection

Non-CF and CF epithelial cells were treated ± human Fas activating antibody (clone CH-11, 0.5 µg/ml, Merck Millipore, Darmstadt, Germany) for 24 hr. Cellular apoptosis (DNA fragmentation) was quantified using the TiterTACS™ Colorimetric Apoptosis Detection Kit (Trevigen, Gaithersburg, MD). Samples were fixed and labelled as per manufacturer's instructions. The Abs_{630 nm} of the wells was measured in a 96-well microplate reader (Synergy HT using Gen5™ software, BioTek UK).

Preparation of whole cell lysates - caspase activity assays and Western blotting

Cells were lysed on ice in lysis buffer (50 mM Tris-HCL pH7.5, 150 mM NaCl, 5 mM EDTA, 0.2% Igepal) for caspase activity assays or in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitor cocktails (Roche Applied Science,

UK) for Western blotting [18]. Cell lysates were centrifuged at 13,000 x g for 10 min to remove insoluble debris and stored at -80°C until required. Total protein concentrations were determined using the BCA method (Pierce BCA Assay, Fisher Scientific, Leicestershire, UK). Cellular caspase activity was determined using a panel of fluorogenic substrates: Ac-Asp-Glu-Val-Asp-7-Amino-4-methylcoumarin (Ac-DEVD-AMC) for caspase-3 and Ac-Ile-Glu-Thr-Asp-AMC (Ac-IETD-AMC) for caspase-8 (Enzo Life Sciences, Inc., Exeter, UK). Samples were incubated with substrate (50 µM) and fluorescence (substrate turnover) was determined by excitation at 360 nm and emission at 460 nm in a 96-well microplate reader (Synergy HT using Gen5™ software, BioTek UK). The rate of substrate hydrolysis was monitored at 37°C every 60 seconds for 1 hr and results were expressed as relative fluorescence unit (RFU)/min and normalised to protein concentration. For Western blotting, denatured samples were separated by electrophoresis on SDS-polyacrylamide gels, transferred to nitrocellulose membrane and probed using rabbit anti-caspase-3 (Cell Signalling), caspase-8 (Cell Signalling), rabbit anti-Bid (Cell Signalling), mouse anti-Fas or rabbit anti-GAPDH (Santa Cruz Biotechnology Inc.). Binding was detected using the appropriate horseradish peroxidase-conjugated secondary antibodies (Fisher Scientific UK), visualized by chemiluminescence (GE Healthcare UK, Buckinghamshire) and analysed using the Syngene G:Box and GeneSnap software (SynGene UK, Cambridge).

RT-PCR

Cell line total RNA was isolated and reverse transcribed as previously described [18]. This was carried out using primers (Invitrogen) for caspase-3 (sense 5'-TTCAGAGGGGATCGTTGTAGAAGTC-3'; antisense 5'-CAAGCTTGTCGGCATACTGTTTCAG-3'), Fas (sense 5'-TAGAAGCTTCGGAGGATTGCTCAACAAC-3'; antisense 5'-

TAGGAATTCTTGGTATTCTGGGTCCG-3') and GAPDH (sense 5'-GAAGGTGAAGGTCGGAGTCA-3'; antisense 5'-TTCACACCCATGACGAACAT-3'). After a hot start, the amplification profile was 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 68°C, and 1 min extension at 72°C for caspase-3; 25 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for Fas; 25 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C for GAPDH. A no cDNA control containing no template was included in all experiments to ensure no genomic contamination. The PCR products were analysed by electrophoresis on 1-2.5% agarose gels containing ethidium bromide and images were captured using Syngene GeneSnap and GeneTools software on a ChemiDoc system.

Statistical analysis

Statistical analyses were carried using GraphPad Prism® software (GraphPad Software, Inc., California, USA). Results are expressed as mean ± SEM. Means were compared by unpaired *t*-test, Mann Whitney test, one-way analysis of variance (ANOVA) or Kruskal-Wallis test as appropriate. A P value of < 0.05 was considered to represent a statistically significant difference (* P < 0.05; ** P < 0.01; *** P < 0.001). Experiments were performed a minimum of three times.

Results

Evaluation of baseline apoptosis and caspase-3 activity in CF and non-CF epithelial cells

Using a commercial assay, relative levels of apoptosis (DNA fragmentation) were found to be significantly elevated in both CF tracheal (CFTE, Figure 1A) and bronchial (CFBE, Figure 1B) epithelial cells compared to corresponding non-CF control cell lines (HTE and HBE). Caspase-3 activity was detected using the fluorogenic substrate Ac-DEVD-AMC and was significantly increased in both CFTE and CFBE cells compared to corresponding healthy control cell lines (HTE and HBE) (Figure 1C, D). No difference in caspase-3 mRNA expression was detected (Figure 1E) suggesting the increased caspase-3 activity observed in CF epithelial cells may be a post-translational alteration in caspase-3 activity or activation of pre-existing caspase-3, and may be related to dysfunctional CFTR. Furthermore, increased cleaved caspase-3 protein in CFTE and CFBE versus HTE and HBE cell lysates was confirmed by Western blot (Figure 1F). Proliferation rates were not found to vary in CFBEs versus HBEs and CFTEs versus HTEs indicating that alterations in proliferation do not contribute to changes in baseline apoptosis and caspase activity observed in CF versus non-CF cell lines (data not shown).

Increased active caspase-8 contributes to increased caspase-3 activity in CF epithelial cells

Caspase-8 activity was analysed in whole cell lysates using the caspase-8 substrate Ac-IETD-AMC and was found to be significantly elevated in CF tracheal (Figure 2A) and bronchial (Figure 2B) epithelial cells compared to non-CF controls. In order to confirm the presence of increased functional, active caspase-8 in CF epithelial cells, we subsequently examined the status of tBID, a substrate for caspase-8, by Western blotting [19]. As illustrated in Figure 2C, increased amounts of tBID were detectable in both CFTE and CFBEs compared to their

respective non-CF control cells confirming the presence of active caspase-8 in CF epithelial cells. In addition, evidence of increased cleaved caspase-8 in CFTE and CFBE versus HTE and HBE cell lysate was demonstrated by Western blot (Figure 2D). Another well-known downstream target of active caspase-8 is caspase-3 and effects of caspase-8 inhibition on caspase-3 activity were also analysed [20]. Treatment of CFTE (Figure 2E) and CFBE (Figure 2F) with the caspase-8 inhibitor Ac-IETD-CHO resulted in a significant decrease in the levels of caspase-3 activity which was observed in both cell types. Overall, these findings suggest that caspase-8 is active and functional in CF tracheal and bronchial epithelial cells compared to non-CF controls.

Investigating the relationship between caspase activity and CFTR function

To investigate the relationship between upregulated caspase-3 activity in CF epithelial cell lines and dysfunctional CFTR, we incubated non-CF control tracheal (Figure 3A) and bronchial (Figure 3B) epithelial cell lines and primary NHBs with the thiazolidinone compound CFTR_{inh-172} in order to induce a 'CF-like' phenotype in these cells (Figure 3C). Following a 24 hr incubation, significantly increased levels of caspase-3 activity were detected in non-CF epithelial cells treated with CFTR_{inh-172}.

Up-regulated Fas expression and activation in CF epithelial cells

Fas mediates signals for cell death through the extrinsic pathway and previous work suggests that the status of CFTR can alter Fas levels and activity [10,21]. Subsequently, Fas expression was explored in CF epithelial cells. As illustrated in Figure 4A, the Fas mRNA was upregulated in both CF tracheal and bronchial cells compared to non-CF control cells. In agreement, protein levels of Fas were also up-regulated in CF epithelial cells (Figure 4B). To

investigate the role of CFTR dysfunction in the control of Fas expression, non-CF NHBE cells were treated with CFTR_{inh}-172 for 24 hr and Fas mRNA and protein levels analysed. Following CFTR inhibition, both Fas mRNA (Figure 4C) and protein (Figure 4D) levels increased compared to untreated cells. In addition, significantly increased Fas mRNA levels were also significantly detected *in vivo* in bronchial brush samples obtained from patients with CF compared to non-CF controls (Figure 4E). These findings suggest that CFTR dysfunction contributes to an increase in the levels of Fas mRNA and protein in CF epithelial cells.

Increased susceptibility of CF epithelial cells to Fas-induced apoptosis

The role of Fas in inducing elevated levels of caspase-3 activity detected in CF epithelial cells was investigated. CF epithelial cells were treated with an anti-Fas neutralising antibody (clone ZB4) or isotype control antibody and the effects on caspase-3 activity were evaluated by fluorogenic activity assay. As depicted in Figure 5, neutralisation of Fas resulted in a significant decrease in caspase-3 activity in both CFTE (Figure 5A) and CFBE (Figure 5B) compared to treatment with isotype control antibody. Following treatment of epithelial cells with a human Fas activating antibody (CH-11), whole cell lysates were analysed for levels of apoptosis and caspase-3 activity. Exposure of epithelial cells to CH-11 resulted in the upregulation of apoptosis (DNA fragmentation), however, this upregulation was significantly higher in both the CFTEs (Figure 5C; 390% vs 161%, $P < 0.05$) and CFBEs (Figure 5D; 218% vs 145%, $P < 0.05$) compared to corresponding control cells. A similar trend was observed for CH-11-induced caspase-3 activity. Following CH-11 treatment, both CFTEs (Figure 5E; 228% vs 114%, $P < 0.01$) and CFBEs (Figure 5F; 192% vs 142%, $P < 0.05$) displayed significantly increased levels of caspase-3 activity compared to non-CF controls.

Discussion

In this study we demonstrate that CF epithelial cells exhibit features of apoptosis in their basal, unstimulated state including increased caspase (-3 and -8) activation compared to non-CF epithelial cells. Analysis of factors responsible for this basal activation revealed upregulated Fas expression and protein levels in CF cells. Upon activation with the CH-11 (Fas activating) antibody there was a more pronounced increase in apoptosis in CF cells compared to non-CF cells with significant upregulation of apoptosis and caspase activity. These data indicate that CF epithelium is in a 'primed' state of activation in the basal state and becomes more overtly apoptotic upon activation. However, it is clear that other factors are involved in upregulated caspase-3 activity in CF epithelial cells as inhibition of Fas signalling (Figure 5) did not completely inhibit caspase-3 activation in our CF cell lines.

Evidence of ER stress has been demonstrated in CF epithelium [22]. However, we did not detect any sign of ER stress in our cells (data not shown) although it has to be stated that our studies focused primarily on unstimulated cells in contrast to the previous ER stress studies in CF. We have observed increased expression of Fas in CF cells compared to non-CF cells. We also evaluated Fas ligand (FasL) expression in our cell lines but did not observe evidence of increased FasL expression in the CF cells (data not shown). The Fas receptor is a death receptor present on the cell surface that can be activated resulting in programmed cell death. Fas forms the death-inducing signaling complex (DISC) upon ligand binding and contains a death effector domain (DED) near its amino terminus which facilitates binding to the DED of caspase-8 [23]. It has previously been demonstrated that Fas expression is increased in conjunctival epithelial cells from CF patients compared to non-CF patients [21]. Another study showed that Fas expression in CF and non-CF lung tissue was comparable

although this was determined semi-quantitatively by immunohistochemistry [13]. In addition, allelic variants within Fas intron 2 generate functional variants of Fas that can modulate the manifestation of CF disease [24]. Fas expression may be altered by CFTR channel activity in CF epithelial cells which may explain, in part, the mechanism of elevated basal apoptosis in these cells. However, this requires further analysis. In the setting of the lung, increased apoptosis resulting from inhalation of anti-Fas antibody in rodents results in significantly increased lung inflammation [25]. Likewise, instillation of the pro-apoptotic compound, ceramide, results in increased apoptosis in the lungs of mice leading to development of emphysema [26].

Our finding of altered apoptosis in CF epithelial cells has the potential to impact on epithelial integrity in the lung although it should be pointed out that others have observed altered effects on apoptosis in CF epithelium during *Pseudomonas* infection [4,27–29] and conditions of oxidative stress [7,30]. Therefore, it seems likely that alterations in apoptosis (decreased or increased) are dependent on the environment and/or stimuli present in the CF lung and may depend on the presence of specific factors of epithelial and bacterial origin. In the context of our findings, more work is required to explain why Fas expression is increased in CF cells and how activation of this receptor leads to increased apoptosis in CF epithelial cells. Further analysis of other cell signalling pathways involved in apoptosis in CF cells is also warranted including analysis of both extracellular factors that could influence the extrinsic pathway as well as factors regulating the intrinsic apoptosis pathway. In conclusion, our data demonstrates that alterations in apoptosis in epithelial cells is basally increased in CF epithelium and requires further investigation for assessment of impact on infection and inflammation in the CF airways.

Acknowledgements

Funding for this study is gratefully acknowledged from the Department of Employment and Learning (QC) and the Northern Ireland Chest Heart and Stroke Association.

Figure Legends

Figure 1. Increased basal apoptosis and caspase-3 levels in CF epithelial cells.

Baseline apoptosis (DNA fragmentation) was detected in non-CF (HTE, HBE) and CF (CFTE, CFBE) **(A)** tracheal and **(B)** bronchial epithelial cells using the TiterTACS™ Colorimetric Apoptosis Detection Kit. The results are expressed as a % of the respective non-CF control (n=6 for each group). Caspase-3 activity was detected in non-CF and CF **(C)** tracheal and **(D)** bronchial epithelial cell lines using the fluorogenic substrate Ac-DEVD-AMC. Results are expressed as the relative fluorescence units (RFU) per min normalised to protein concentration (n=6 for each group). **(E)** Caspase-3 and GAPDH mRNA expression were detected by RT-PCR (representative agarose gel from n=3). **(F)**. Cleaved caspase-3 protein was detected by Western blot (representative Western blot from n=3).

Figure 2. Increased caspase-8 activity in CF epithelial cells activates caspase-3.

Caspase-8 activity was analysed in **(A)** tracheal and **(B)** bronchial epithelial cell whole cell lysates using the fluorogenic Ac-IETD-AMC substrate. Results are expressed as the relative fluorescence units (RFU) per min normalised to protein concentration (n=6 for each group). **(C)** Cleavage of Bid to its truncated form (tBid, 15 KDa) was detected by Western blotting of non-CF and CF tracheal (HTE, CFTE) and bronchial (HBE, CFBE) epithelial cell whole cell lysates. GAPDH was used as a loading control. **(D)** Caspase-8 protein was detected by Western blot (representative Western blot from n=3). Caspase-3 activity detected in **(E)** CFTEs and **(F)** CFBEs treated in the absence or presence of a caspase-8 inhibitor (Casp8-inh, Ac-IETD-CHO, 10 µM) for 24 hr. Cells were lysed and caspase-3 activity was detected using Ac-DEVD-AMC. Results are expressed as a % of untreated control cells (n=6 for each group).

Figure 3. Effects of CFTR inhibition on epithelial cell caspase-3 activity.

(A) Non-CF tracheal (HTE), **(B)** bronchial (HBE) and **(C)** primary human tracheal/bronchial epithelial cells (NHBE) were treated in the absence or presence of a CFTR inhibitor (CFTR_{inh}-172 20 μ M) for 24 hr. Caspase-3 activity was detected in whole cell lysates using the fluorogenic substrate Ac-DEVD-AMC. Results are expressed as a % of respective untreated control cells.

Figure 4. CF epithelial cells express increased levels of Fas (CD95).

(A) Fas mRNA expression was detected in tracheal and bronchial epithelial cell lines by RT-PCR. GAPDH was used as a loading control (representative agarose gel from n=4). **(B)** Western blotting of Fas and GAPDH in tracheal and bronchial epithelial cell line whole cell lysates (representative Western blot from n=4). Non-CF primary human tracheal/bronchial epithelial cells (NHBE) were treated in the absence or presence of a CFTR inhibitor (CFTR_{inh}-172; 20 μ M) for 24 hr and the effects on **(C)** Fas mRNA and **(D)** Fas protein detected by RT-PCR and Western blotting, respectively. **(E)** Fas mRNA expression in CF and non-CF control bronchial brushings (n=3) were quantified by qRT-PCR as described in the methods.

Figure 5. Inhibition of Fas down-regulates caspase-3 activity in CF epithelial cells.

(A) CFTEs and **(B)** CFBEs were treated with isotype control or anti-Fas neutralising antibody (clone ZB4, 100 ng/ml) for 6 hr. Cells were lysed and caspase-3 activity measured using the fluorogenic substrate Ac-DEVD-AMC. Results are expressed as a % of untreated controls. * P < 0.05. CF and non-CF tracheal and bronchial epithelial cell lines were stimulated with the Fas activating antibody CH-11 (0.5 μ g/ml) for 24 hr (n=6 for each group). **(C, D)** Apoptosis (DNA fragmentation) was quantified using the TiterTACS™ Colorimetric Apoptosis

Detection Kit. Results are expressed as a % of respective untreated control and groups were compared by one way ANOVA (n=8 for each group). **(E, F)** Caspase-3 activity was detected using the Ac-DEVD-AMC fluorogenic substrate. Results are expressed as a % of respective untreated control and groups were compared by one way ANOVA (n=8 for each group).

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Figure 1

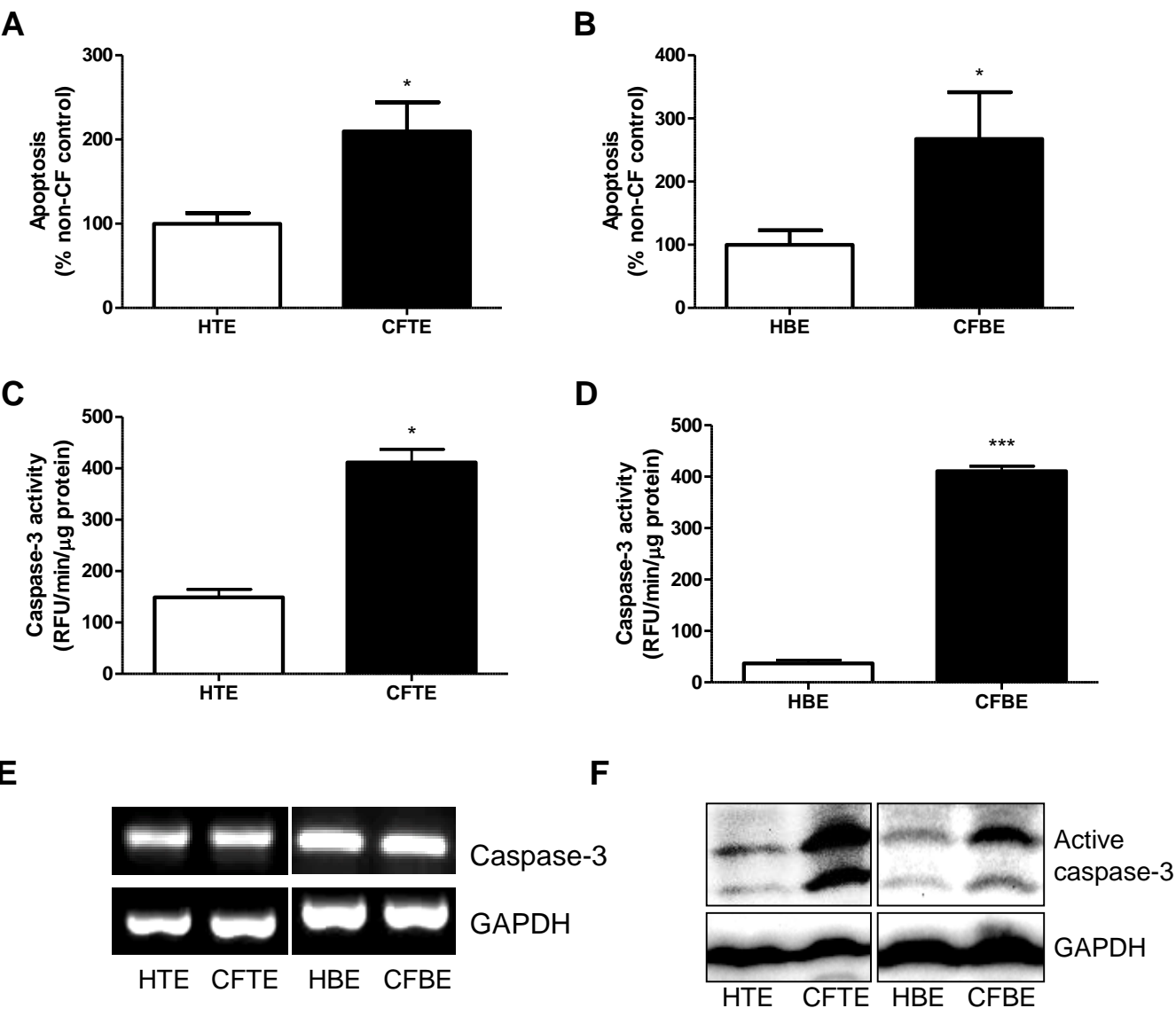
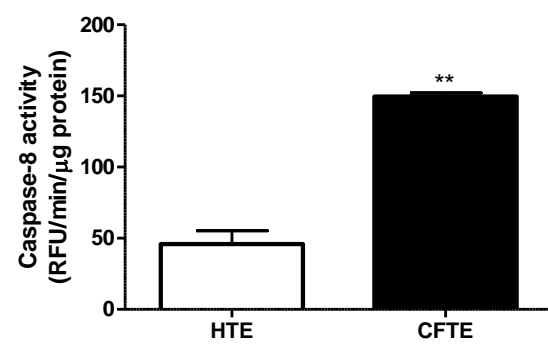
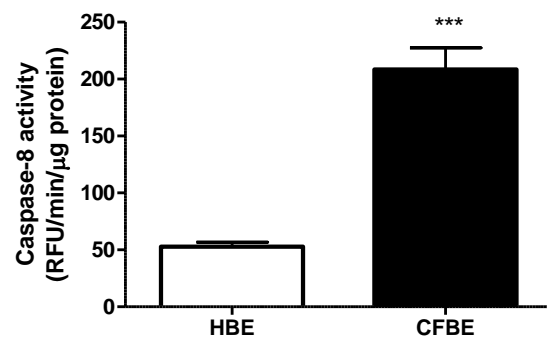


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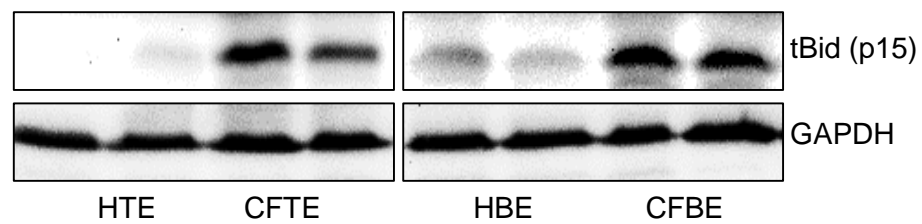
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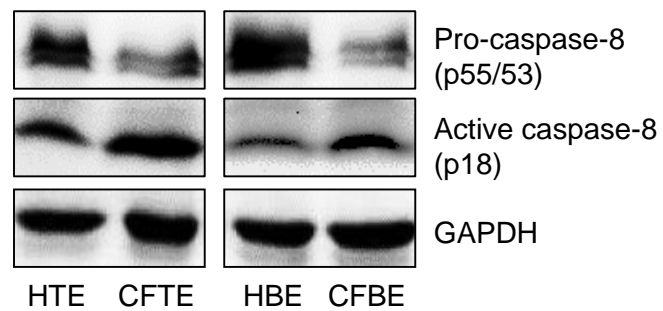
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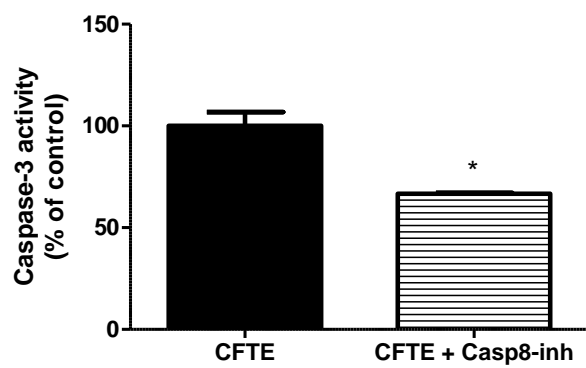
C



D



E



F

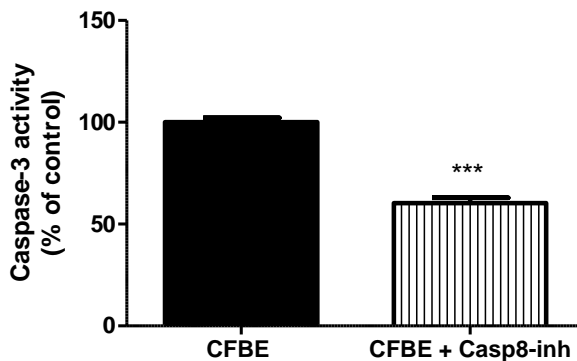


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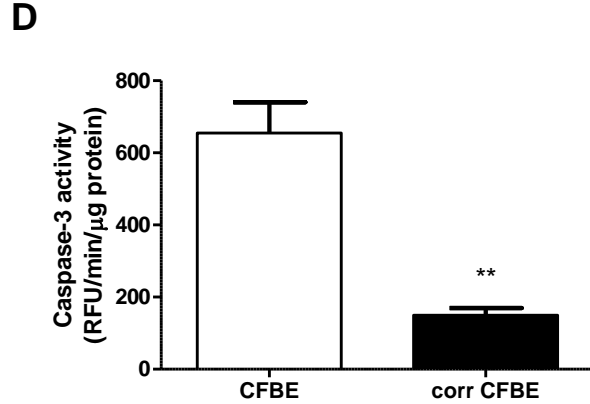
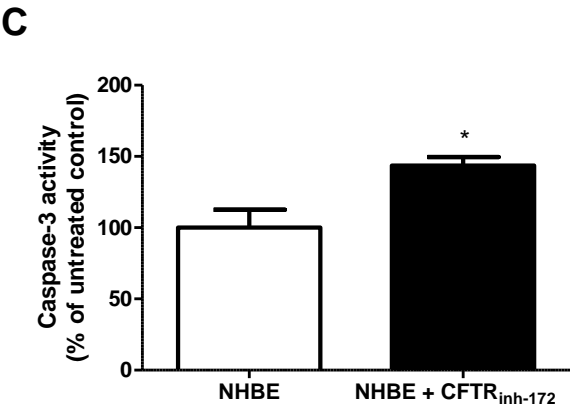
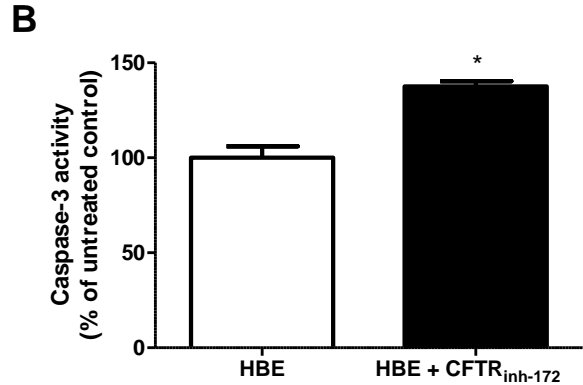
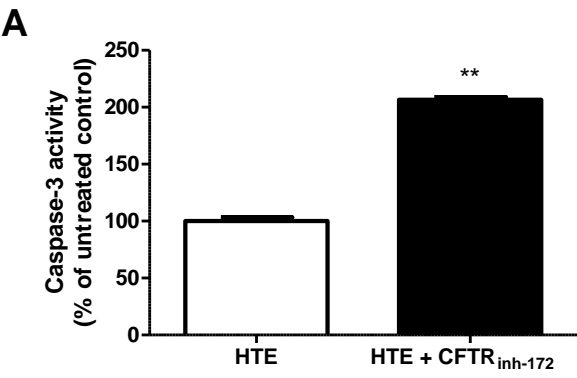
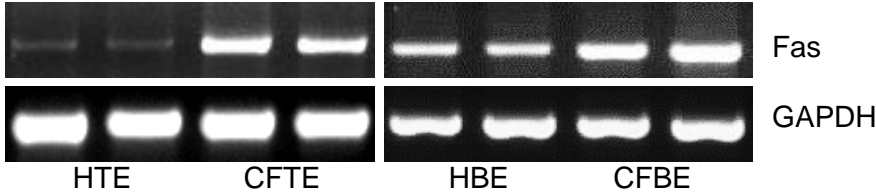
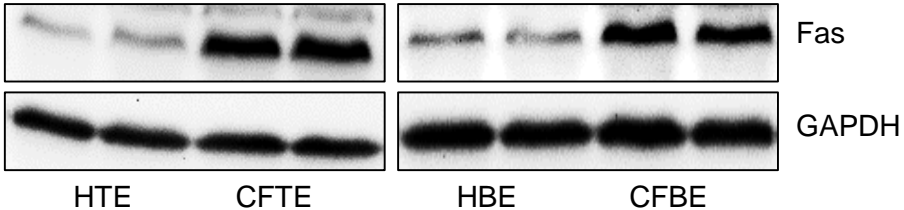


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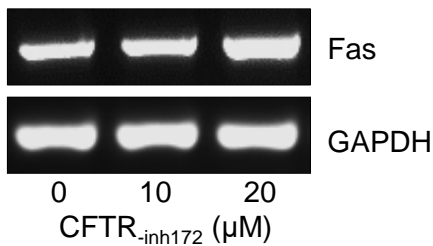
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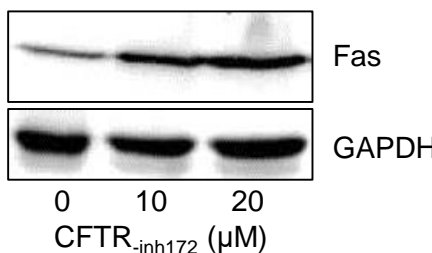
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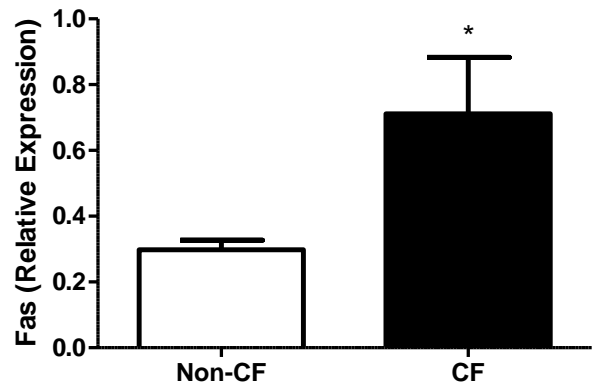


Figure 5

